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A new candidate mutation, G1629R, in a patient with type 2A von Willebrand's disease: basic mechanisms and clinical implications

A B S T R A C T

Background and Objectives. Type 2A von Willebrand's disease (VWD) refers to disease variants with decreased platelet-dependent function of von Willebrand factor (VWF) associated with the absence of high molecular weight (HMW) multimers. The candidate G1629R mutation, identified in an Italian patient with type 2A VWD, was expressed to confirm the relationship between phenotype and genotype.

Design and Methods. Plasma samples from the patient were studied after 1-deamino-8-D-arginine vasopressin (DDAVP) or FVIII/VWF concentrate injections. Furthermore, an expression vector carrying the G1629R mutation was constructed by site-directed mutagenesis and transiently expressed in Cos-7 cells. The characteristics of the corresponding recombinant protein were analyzed.

Results. After DDAVP infusion, factor VIII and VWF activities increased and HMW VWF multimers were transiently observed in the patient's plasma. VWF activity increased only after administration of a dual FVIII/VWF concentrate. ADAMTS-13 activity did not change significantly before or after the therapies. Secretion, in culture medium, of the corresponding mutated protein (R1629-rVWF) was slightly decreased and this rVWF contained intermediate and HMW multimers. Furthermore, binding of R1629-rVWF to platelet *GPIb* was moderately reduced compared to that of the wild-type rVWF.

Interpretation and Conclusions. Based on the DDAVP and *in vitro* expression results, we classified the G1629R mutation in group 2 type 2A mutations. Our findings could explain why DDAVP may only be partially effective and suggest that FVIII/VWF concentrates should be used in cases of prolonged mucosal bleeding and major surgery when functional VWF is required.

Key words: von Willebrand factor, von Willebrand's disease, mutation, recombinant protein, Cos-7 cells.

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Von Willebrand factor (VWF) is a large adhesive glycoprotein that plays a critical role in the early phases of hemostasis. It protects factor VIII (FVIII) from proteolytic degradation in the circulation and mediates platelet adhesion at the sites of vessel injury.[1] VWF, synthesized by endothelial cells and megakaryocytes as a 2,813 amino acid pre-pro-polypeptide, is modified by post-translational processing including propeptide (amino acids 23-763) cleavage and multimerization.[2] Thus, VWF circulates in plasma as a series of disulphide-linked multimers ranging from 0.5 to over 10^7 daltons. Some plasma VWF subunits appear to be modified by ADAMTS-13, which induces a specific proteolytic cleavage between amino acid residues Y1605 and M1606 in the A2 domain.

VWF defects result in von Willebrand's disease (VWD), the most common inherited bleeding disorder in humans. This disorder can result from either a quantitative (types 1 and 3) or qualitative (type 2) defect in VWF. Patients with type 2A VWD have decreased VWF platelet-dependent function associated with the absence of high molecular weight (HMW) VWF multimers.[3] Most of the mutations responsible for this sub-type have been identified in the A2 domain clustered within the 134 amino acid sequence from G1505 to E1638 (*http://www. sheffield.ac.uk/vwf*). Expression of recombinant VWF (rVWF) containing these mutations has elucidated two pathogenic mechanisms leading to the characteristic loss of HMW multimers in plasma VWF.[4] Group 1 type 2A VWD mutations impair the biosynthesis and/or secretion of VWF multimers and lead to the retention of mutated VWF in the endoplasmic reticulum and subsequent decrease in large and intermediate VWF forms in both plasma and

platelets. In group 2 type 2A VWD, VWF is normally processed and secreted but is more sensitive to proteolysis and thus the levels of HMW VWF multimers in plasma are decreased. Platelet VWF in patients belonging to this group is protected from plasma protease and is, therefore, normal. Furthermore, Englender *et al.*[5] have shown that the V1665E mutation represents a new group of type 2A mutations characterized by a striking degree of intracellular proteolysis.

We report here the phenotypic data from an Italian patient with type 2A VWD, characterized by a mutation changing glycine 1629 into arginine (G1629R). To understand this VWF defect better, VWF activities were measured before and after infusion of 1-deamino-8-D-arginine vasopressin (DDAVP) and transfusion of a plasma-derived dual FVIII/VWF concentrate. The phenotypic data of the patient were compared with those obtained *in vitro* for the recombinant mutant VWF obtained after site-directed mutagenesis and transient expression in Cos-7 cells. Both *ex vivo* and *in vitro* data suggest that the G1629R mutation can be classified in group 2 type 2A mutations.

Design and Methods

Clinical and phenotypic characterization of the patient

This study was performed in an Italian patient with type 2A VWD diagnosed at the Angelo Bianchi Bonomi Hemophilia Thrombosis Center of Milan, also included in the European project entitled *Optimizing Orphan Drug Therapy in Severe Forms of VWD.*[6]

The patient had a negative family history but a lifelong personal history of epistaxis, gum and gastrointestinal bleeding and bleeding after dental extractions. Minor bleeding problems had been solved in the past using DDAVP. However, large doses of intermediate purity FVIII/VWF concentrates were necessary to stop gastrointestinal bleeding and prevent hemorrhage during major surgery. Because of previous transfusions received in the 1970s and 1980s, the patient has a degree of liver cirrhosis caused by hepatitis C virus infection.

The patient's bleeding time (BT) was measured using a commercially available device that makes two standard 1 mm horizontal skin incisions in the volar region of the forearm (Simplate IIR, BioMérieux, Inc, Durham, NC, USA). Factor VIII activity (FVIII:C) was evaluated using the pro-coagulant activity test (the one stage assay) derived from an activated partial thromboplastin time (APTT) assay in FVIII-depleted plasma (Hemoliance) by using Electra 1600 Coagulometer produced by Instrumentation Laboratories (IL, Lexington, MA, USA). The reagent for APTT is SyntAsil (Hemoliance)

which is also distributed by Instrumentation Laboratories. VWF antigen (VWF:Ag) (ELISA), VWF ristocetin cofactor activity (VWF:RCo) (by agglutination) and VWF collagen binding capacity (VWF:CB) (ELISA) were determined as described in a recent report.[7] Platelet VWF was measured as described elsewhere.[8] Multimeric analysis of plasma VWF was performed using sodium dodecyl sulfate (SDS) agarose (1.2% or 1.6%) electrophoresis.[9] The gels were scanned and the resulting densitometric profiles were used to calculate the percentage of HMW VWF multimers (> 10 mers). The ristocetin-dependent platelet binding assay on patient's plasma VWF was performed as previously reported.[10] In order to determine the biological response to DDAVP, the patient, after having signed informed consent and when not bleeding, was given a 0.3 μg/kg i. v. infusion of DDAVP under medical supervision. Venous blood was collected at specific times (before, 0.5, 1, 2 and 4 hours after the infusion) according to the standardized protocol.[6] More recently, we also had the opportunity to measure FVIII/VWF and BT after administration of a plasma-derived dual FVIII/VWF concentrate (Alphanate, Alpha-Therapeutics). The patient had not bled during the previous 15 days and was given 40 U/kg of Alphanate before a single dental extraction. To compare data with those collected during the DDAVP trial, blood samples were taken before and 1, 2, and 4 hours after administration of this concentrate. Finally, to determine whether the VWF cleaving protease, ADAMTS-13, was affecting the patient's VWF, we measured ADAMTS-13 activity before and after both therapies according to a method already published.[11]

Detection and expression of VWF molecular abnormality

Exon 28 of the patient's VWF gene was amplified by polymerase-chain reaction (PCR) and sequenced (Genoscreen, Institut Pasteur, Lille, France). A mutated vector containing the G1629R substitution was constructed in a pSVVWFA vector[12] using the Transformer site-directed mutagenesis kit according to the manufacturer's instructions (Invitrogen Life Technologies). After verifying that no undesired mutations were present, the mutated Not I-Nhe I fragment [nucleotide (nt) 2974-5209] was sub-cloned into a normal expression vector. Wild type (WT) and mutated expression vectors were transiently transfected into Cos-7 cells using the diethylaminoethyl dextran method as previously described.[13] Expression was performed in the presence of 10% (V/V) fetal bovine serum (Invitrogen Life Technologies). Culture medium containing rVWF was collected in the presence of 10 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride (PMSF) and cleared from cellular fragments by centrifugation for 5 minutes at 2,500 g. The cell lysate was reconstituted in 1

Table 1. Factor VIII/VWF and ADAMTS-13 activities of the patient before and after DDAVP infusion.

	BT (min)	VWF:Ag (IU/dL)	VWF: RCo (IU/dL)	VWF: CB (*) (IU/dL)	VWF: RCo/Ag ratio	VWF: CB/Ag ratio	ADAMTS-13 (*) (IU/dL)	FVIII:C (IU/dL)
Before infusion	> 30	100	30	4	0.30	0.04	44	61
0.5 hr post-infusion	n. d.	190	67	18	0.35	0.25	n. d.	192
1 hr post-infusion	10	236	79	26	0.33	0.19	32	203
2 hr post-infusion	20	232	60	25	0.26	0.11	38	203
4 hr post-infusion	> 30	252	62	15	0.25	0.06	39	163

Note that VWF:CB and ADAMTS-13 were measured more recently, in frozen samples.

Table 2. Factor VIII/VWF and ADAMTS-13 activities of the patient before and after exogenous VWF given by 40 U/kg of a dual FVIII/VWF concentrate (Alphanate).

	BT (min)	VWF:Ag (IU/dL)	VWF: RCo (IU/dL)	VWF: CB (IU/dL)	VWF: RCo/Ag ratio	VWF: CB/Ag ratio	ADAMTS-13 (IU/dL)	FVIII:C (IU/dL)
Before infusion	> 30	84	26	3	0.31	0.04	48	91
1 hr post-infusion	n. d.	170	107	88	0.63	0.52	43	175
2 hr post-infusion	8	180	80	67	0.44	0.37	52	156
4 hr post-infusion	n. d.	174	72	55	0.41	0.32	56	151

mL lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 8.0, 5 mM EDTA, 1% Nonidet P-40, 2 mM N-ethyl-maleimide, 10 mM benzamidine, 1 mM PMSF) and centrifuged at 2,500 g for 5 minutes before use.

rVWF analysis

rVWF in the media and in cell lysates from transfected cells was quantified by ELISA.[13] The multimeric composition of rVWF was analyzed in 1.5% agarose gels in the presence of 0.1% SDS and the VWF multimers were visualized with alkaline phosphatase-conjugated anti-VWF polyclonal antibodies.[13] Ristocetin-dependent platelet binding assays were performed as previously reported.[10]

Results

Patient's phenotypic data

The BT with FVIII/VWF and activity of ADAMTS-13 were measured on two different occasions: before and after DDAVP (Table 1) and before and after a FVIII/VWF

concentrate (Table 2). At baseline, the patient showed normal FVIII:C and VWF:Ag levels but a low VWF:RCo/Ag ratio (0.3) with even lower values of VWF:CB/Ag (0.04). A decrease in HMW and intermediate molecular weight plasma VWF multimers was observed by agarose gel electrophoresis (Figure 1A) with an increase in the intensity of the flanking bands in the triplet structure (Figure 1B). There was almost no binding of plasma VWF to GPIb (Figure 2), in agreement with the very abnormal (>2.5 mg/mL ristocetin) ristocetin-induced platelet aggregation (RIPA) in the patient's platelet-rich plasma (*not shown*). The platelet VWF content was relatively normal with VWF:Ag and VWF:RCo values being 0.75 and 0.33 IU/10[9] platelets, respectively.

One hour after DDAVP infusion, the patient's VWF:Ag level had increased to 236 IU/dL, and the VWF:RCo activity had increased to 79 IU/dL (Table 1). These levels remained almost constant 4 hours after infusion. The BT shortened from > 30 minutes to 10 minutes, 1 hour post-infusion but was again very prolonged 4 hours after the injection (Table 1). The VWF:CB, already lower than VWF:RCo at baseline, remained always lower following
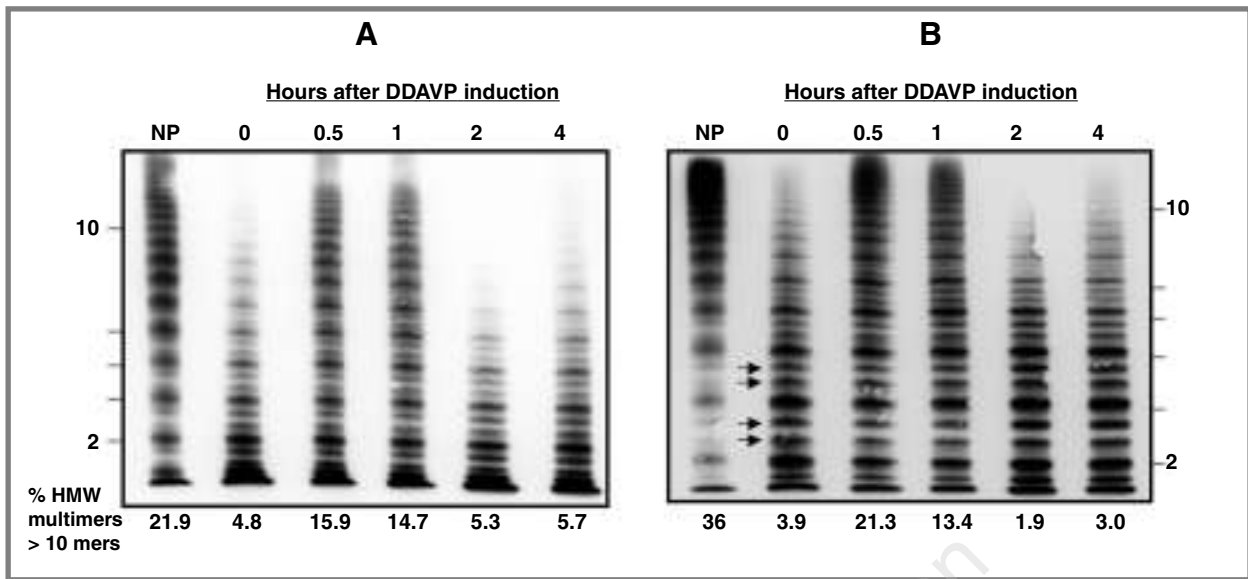
Figure 1. Multimeric structure of plasma VWF in SDS-1.2% agarose gel (A) or SDS-1.6% agarose gel (B). Numbers correspond to the various multimers of the VWF protomer. Arrows indicate VWF satellite bands that are increased in the patient's plasma. NP: normal plasma. The percentage of HMW multimers above 10-mers, determined by densitometric scanning, is indicated below each lane.
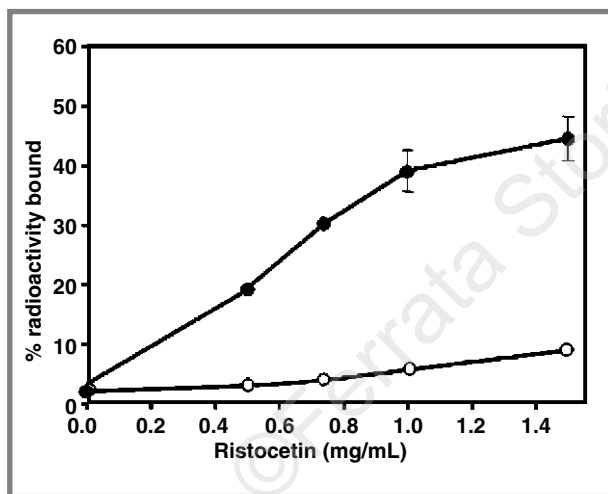

Figure 2. Ristocetin-induced binding of normal plasma (●) or patient's plasma (○) VWF to platelets.

DDAVP. HMW multimers were immediately released into the plasma following infusion, as indicated by a normal plasma VWF multimeric structure observed 0.5 hour post-infusion (Figure 1A). However, this effect was only transient as the VWF multimeric pattern was again abnormal 2 hours post-infusion.

The administration of exogenous VWF in a high-purity dual FVIII/VWF concentrate shortened the BT at two hours (8 min) and normalized both VWF:RCo and VWF:CB (Table 2). The levels of ADAMTS-13 activity were
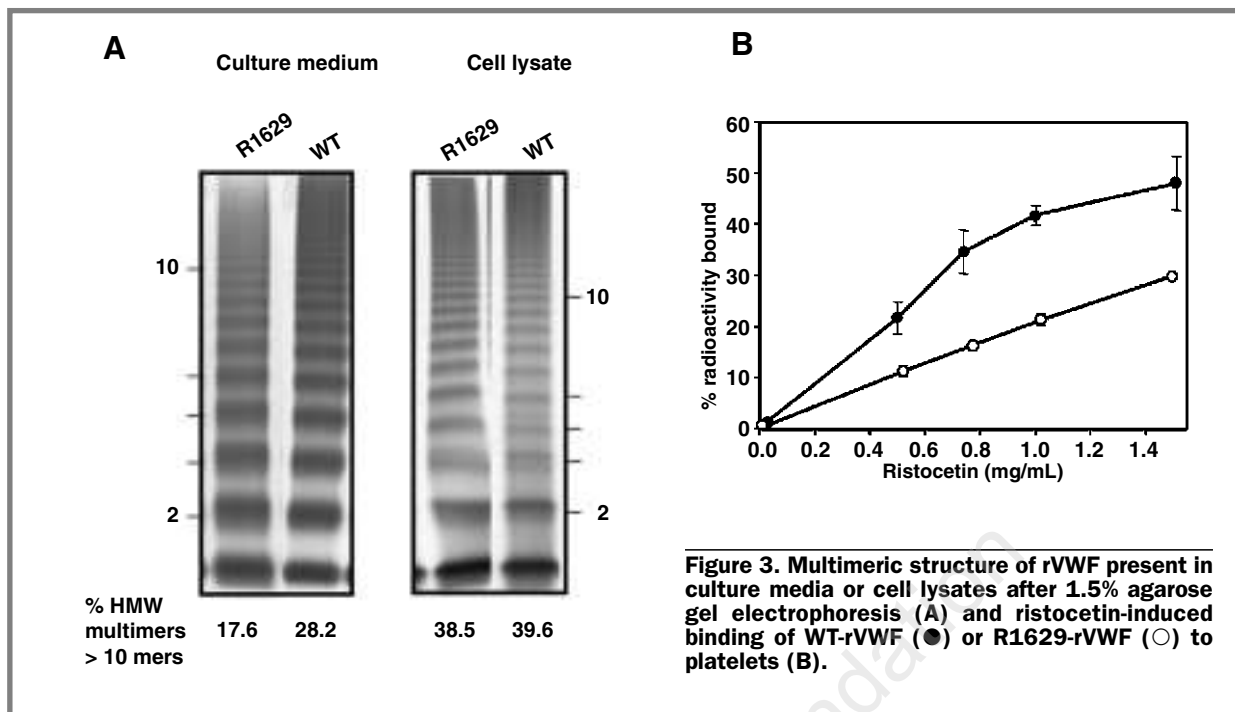
at the lowest limit of normal range (44 and 48%). They decreased slightly just after both treatments and then ranged between 38 and 56%. (Tables 1 and 2).

Identification of the patient's molecular abnormality

Sequencing of PCR-amplified exon 28 of the patient's DNA revealed the presence of a cytosine in addition to the normal guanine at nt 4885. The sequencing profile therefore indicated that the patient was heterozygous for this nt change, resulting in the substitution of an arginine residue for glycine at position 1629 in pre-pro VWF (mutation G1629R).

Expression and characterization of rVWF

Cos-7 cells were transiently transfected with WT or R1629 plasmids (6 transfections of 2 different clones). The levels of mutated rVWF present in the culture media and in the corresponding transfected Cos-7 cell lysates were slightly lower than the levels of the WT-rVWF obtained in the same transfection experiments (78±18% and 77±20%, respectively). As shown in Figure 3A, the multimeric profile of the mutated rVWF present in culture media showed that all multimers were present although a slight decrease in the highest molecular weight multimers was observed. The multimeric structure in the corresponding cell lysates was similar to that of WT-rVWF. Mutated rVWF showed a moderately weakeNED capacity for binding to platelets, when compared to WT-rVWF (Figure 3B).

A Culture medium Cell lysate **B**

R1629 WT R1629 WT

Figure 3. Multimeric structure of rVWF present in culture media or cell lysates after 1.5% agarose gel electrophoresis (A) and ristocetin-induced binding of WT-rVWF (●) or R1629-rVWF (○) to platelets (B).

Discussion

We report here the case of an Italian VWD patient who has a low VWF:RCo/Ag and VWF:CB/Ag ratio, loss of high and intermediate molecular weight plasma VWF multimers and no ristocetin-induced binding of plasma VWF to platelets. The fact that both VWF:RCo and VWF:CB activities were abnormal together with the relative loss of HMW multimers in this patient immediately suggested a type 2A and not a type 2M VWD defect.[14] The platelet VWF content was relatively normal, even though VWF:RCo was lower than VWF:Ag. These VWF abnormalities could be responsible for the very prolonged BT observed on different occasions. Unfortunately, we could not test the PFA-100 in this particular patient; however, in our hands, PFA-100 has correlated very well with BT in other cases of type 2A VWD.[15] Physiologic proteolysis of endogenous VWF is regulated by plasma levels of ADAMTS-13.[16] In our patient, the baseline level of ADAMTS-13 was borderline, probably because of abnormal liver function.[11] There was a slight and transient reduction of ADAMTS-13 activity after the increase of endogenous (DDAVP) or exogenous (FVIII/VWF concentrate) VWF, as recently reported by several authors.[17,18] This patient responded only transiently to DDAVP as indicated by correction of BT, doubling of VWF levels and appearance of HMW multimers in plasma 1 hour post-infusion. However, the BT was again very prolonged after 2-4 hours, consistent with the relative loss of HMW multimers in plasma. Conversely, the use of a

high purity FVIII/VWF concentrate improved both VWF:RCo and VWF:CB activities and almost corrected the BT also after two hours. This suggests that a FVIII/VWF concentrate must be recommended in severe bleeding episodes and for major surgery in such patients.

After sequencing the VWF exon 28, the G1629R substitution was identified in the heterozygous state. This mutation had been previously reported in a Spanish family and classified as type 2A VWD.[19] The Spanish patient was found to be responsive to DDAVP. However, although he showed a discrepancy between VWF:Ag (31 ± 14 IU/dL) and VWF:RCo (4 ± 1 IU/dL) levels, these levels were lower than those in our Italian patient.

Four relatives of the Spanish patient were reported to bleed excessively after surgical surgery and dental extractions. Despite the relatively higher values of FVIII/VWF activities, the Italian patient had a history of very severe bleeding: minor bleedings and surgery had been treated successfully with DDAVP but recurrent episodes of gastrointestinal bleeding and major surgery (e.g. colectomy) had required extensive infusions of FVIII/VWF concentrates. Nevertheless, improvement of VWF levels and hemostasis after DDAVP treatment observed in the propositi of both families suggest that the G1629R mutation is compatible with normal multimerization but could induce increased sensitivity of VWF to extracellular proteolysis.

To confirm the effect of this mutation on the patient's phenotype, full length cDNA carrying the G1629R mutation was transiently expressed in Cos-7 cells. The corresponding secreted rVWF contained well

multimerized multimers although some HMW multimers were decreased, probably related to proteases known to be present in fetal bovine serum. Mutated rVWF present in cell lysates was normal. Binding of secreted R1629-rVWF to platelets was moderately decreased. The results therefore resemble data obtained with the R1597W mutation (a type 2A-group 2 mutation) which induced a normal multimeric pattern of corresponding rVWF although ristocetin-induced binding to platelets was decreased.[20] The fact that the R1629-rVWF binding to GPIb was moderately decreased whereas the patient's VWF binding was almost nil suggests that the functional abnormality of the plasma VWF is the result of the combined effects of both the mutation and the disappearance of HMW multimers.

Using molecular modeling, Jenkins et al.[21] showed that type 2A VWD mutation sites in the A2 domain correspond to buried residues that are otherwise 100% conserved across 28 species analyzed. The glycine residue at position 1629 is also conserved across these 28 species confirming that a precise conformation in this area is required for correct protein structure and function. The G1629R mutation may expose a region on the surface of the protein, resulting in an increased susceptibility of VWF to plasma proteases.

In conclusion, the laboratory data from our patient with a specific mutation within exon 28 of the VWF gene are not only important for understanding the basic mechanisms of type 2A VWD but can also help to define the most appropriate clinical management. In fact, the data show that DDAVP may be only partially effective, especially for prolonged mucosal bleeding episodes and after major surgery. These phenotypic and expression data strongly support the use of concentrates containing VWF in type 2A VWD patients in such clinical settings.

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